Heterologous expression of a plant uracil transporter in yeast: improvement of plasma membrane targeting in mutants of the Rsp5p ubiquitin protein ligase.

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Running title: Functional expression of a plant transporter in yeast

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Keywords: transporters, yeast, heterologous expression, Rsp5p, ubiquitin protein ligase

Abstract:

Plasma membrane proteins involved in transport processes play a crucial role in cell physiology. On account of these properties, these molecules are ideal targets for development of new therapeutic and agronomic agents. However, these proteins are of low abundance, which limits their study. Although yeast seems ideal for expressing heterologous transporters, plasma membrane proteins are often retained in intracellular compartments. We tried to find yeast mutants potentially able to improve functional expression of a whole set of heterologous transporters. We focused on *Arabidopsis thaliana* ureide transporter 1 (AtUPS1), previously cloned by functional complementation in yeast. Tagged versions of AtUPS1 remain mostly trapped in the endoplasmic reticulum and were able to reach slowly the plasma membrane. In contrast, untagged AtUPS1 is rapidly delivered to plasma membrane, where it remains in stable form. Tagged and untagged versions of AtUPS1 were expressed in cells deficient in the ubiquitin ligase Rsp5p, involved in various stages of the intracellular trafficking of membrane-bound proteins. *rsp5* mutants displayed further plasma membrane stabilization of untagged AtUPS1, and improved steady state amounts of tagged versions of AtUPS1. *rsp5* cells are thus powerful tools to solve the many problems inherent in heterologous expression of membrane proteins in yeast, including ER retention.

Introduction:

Systematic sequencing of the genomes of complex organisms (e.g., the plant *Arabodopsis thaliana*, certain parasites, and man) has revealed a great number of genes that are likely to code for membrane transporters [1]. Many of these proteins are assigned to this functional category on the sole basis of sequence similarity to known transport proteins. Their biochemical properties are thus unknown and information regarding substrate specificity is often lacking. To possess such knowledge would be important both scientifically and industrially, because transport proteins (channels, pumps, and carriers) play a crucial role in cell physiology. This is illustrated by the numerous genetic diseases that are caused by defective transport systems. Furthermore, cell surface membrane transporters may be ideal targets for the development of new therapeutic or agronomic agents. Biological systems are thus required for the functional expression of each transport protein and for the study of its biochemical properties by means of quick, simple tests. The yeast *Saccharomyces cerevisiae* expression system best meets these requirements. It is easy to use and inexpensive, its "transportome" has been extensively analyzed *in silico* [2,3] and the function of over 150 yeast transport proteins has been identified [4]. Moreover, the construction of a complete collection of strains deleted for each of the 6200 yeast genes [5] led to an impressive number of *S. cerevisiae* mutants deficient in the transport of compounds as diverse as inorganic ions, metabolites, and drugs. The phenotypes of these mutants are easily identified after growth on solid media and have been used extensively in complementation tests, notably to clone and characterize heterologous transporters.

Hundreds of plant transporters have been successfully cloned by complementation in yeast [6-9].The yeast system has also been used in biochemical approaches, for example, to analyze the functional properties of plant H^+-ATPases [10]. Nevertheless, although yeast seems ideal for expressing plasma membrane proteins including transporters and receptors, investigators often found some of these proteins to be inactive in yeast, sometimes because the protein did not fold properly and/or was not delivered to the plasma membrane but accumulated in a secretory compartment (endoplasmic reticulum (ER), Golgi apparatus, secretory vesicles), sometimes even leading to formation of karmellae [11]. On the other hand, some heterologous proteins correctly delivered to the plasma membrane have been described to undergo rapid endocytosis and turnover, resulting in low plasma membrane levels [12].

A few attempts to improve functional expression in yeast of heterologous plasma membrane transporters have been reported, but they often focused on only one given transporter [13,14]. Attempts to identify yeast mutants that would potentially improve the functional expression of a whole set of plasma membrane proteins and more specifically transporters, are still lacking. Finding such mutants for improvement of the functional expression of several plant and mammalian transporters was the objective of an European network, EFFEXPORT ("Engineering yeast for efficient expression of heterologous membrane transporters"). We report here data we obtained within EFFEXPORT in the case of the *Arabidopsis Thaliana* ureide transporter (AtUPS1), already known to be functionally expressed in *S. cerevisiae* [15]. AtUPS1, was identified by functional complementation of a yeast *dal4 dal5* mutant [15] defective in uptake of allantoin. AtUPS1 belongs to a superfamily of plant transporters with five members in Arabidopsis. Analysis of hydrophobicity

predicts 10 putative transmembrane domains, with N- and C-termini predicted to protrude into the extracellular space. UPS proteins display a conserved central domain between predicted transmembrane helices 5 and 6, which contains a consensus sequence for a P loop, also designated as a « Walker A » motif for ATP binding [15]. Expression in yeast and Xenopus oocytes allowed to demonstrate that AtUPS1 mediates uptake of allantoin and related metabolites including uracil [15,16]. The fate of AtUPS1 can thus be compared to that of the endogenous uracil permease (Fur4p), a well-known yeast transporter, the trafficking of which has been studied extensively (reviewed in [17]). Fur4p, which belongs to a family of five homologous proteins in *S. cerevisiae* [2], also consists of ten transmembrane spans, with cytoplasmic oriented N- and C- termini [18]. Like most yeast plasma membrane proteins, it displays plasma membrane ubiquitylation, catalyzed by the Rsp5p ubiquitin protein ligase, a modification triggering its internalization and subsequent vacuolar degradation [19]. The same ubiquitin ligase, the sole member of the family of Nedd4 ubiquitin ligases in yeast [20], was also demonstrated to be required for direct Golgi-to-vacuole trafficking of a number of plasma membrane transporters, including Fur4p [17,21], *i.e.* proteins routed to a direct degradation pathway bypassing the plasma membrane under certain nutrient/substrate conditions. Rsp5p was also described to be required for Golgi-to-vacuole traffic of misfolded plasma membrane proteins misrouted to the vacuole [22]. In latter two cases, these proteins were retargeted to plasma membrane in *rsp5* mutants. *rsp5* mutants thus display increased steady state amounts of many plasma membrane transporters (reviewed in [17]), and were good candidates for potential improvement of functional expression of heterologous transporters. However, in addition to its role in trafficking of membrane proteins [17], Rsp5p has many other functions, including essential

functions [23,24]. Hence, only defined *rsp5* mutants may be used for optimization of functional expression of heterologous proteins. Two viable *rsp5* mutants that were described to be affected for several of the trafficking functions of Rsp5p appeared interesting: *npi1* mutant, with altered *RSP5* promoter, leading to a 10-fold reduction in the steady state amount of this protein [25], and *rsp5ΔC2,* lacking Rsp5p C2 domain involved in localization of the enzyme at plasma membrane and endosomes [26].

We report here an analysis of the fate of AtUPS1, as compared to that of Fur4p, in wild type and *rsp5* mutant cells, and show that *npi1* and *rsp5ΔC2* cells improved functional expression of the plant transporter.

Results

AtUPS1 mediates high affinity uracil transport in yeast

As outlined above, when expressed in yeast, AtUPS1 transports [^{14}C] labelled allantoin with high affinity and potentially other heterocyclic compounds as suggested by competition studies [15]. Moreover, AtUPS1 mediates uracil uptake when expressed in Xenopus oocytes [16]. In order to monitor the intracellular fate of AtUPS1 in yeast, *UPS1* was cloned in a multicopy plasmid under the control of the galactose-inducible GAL promoter. We defined the characteristics of uracil uptake mediated by GAL-*UPS1*, as compared to uracil uptake mediated by the endogenous Fur4p cloned under the control of the same promoter. *fur4Δ* cells grown on galactose expressing either GAL-*UPS1* or GAL-*FUR4* displayed high sensitivity to 5-fluorouracil (5FU), a toxic analog of uracil: cells expressing the transporters were unable to grow on plates containing 1μM 5FU, whereas cells transformed with a control plasmid

grew normally (Fig 1A). To determine the uracil transport properties of AtUPS1 quantitatively, radiotracer uptake studies were performed using [^{14}C] uracil. [^{14}C] uracil uptake mediated by GAL-*UPS1* expressed in *fur4Δ* cells growing exponentially and fully induced on galactose was linear for at least 3 min, concentration-dependent, and displayed saturation kinetics with an apparent Km of 6 μM, close to that observed in parallel for cells expressing GAL-*FUR4* (7.5 μM). *fur4Δ* cells expressing GAL-*UPS1* grown overnight in galactose containing media displayed an activity 50% of cells expressing GAL-*FUR4, i.e.*, more than 30-fold that of chromosomal encoded *FUR4* (Fig. 1B), thus providing a sensitive assay to follow AtUPS1 intracellular fate.

Insight into AtUPS1 intracellular trafficking in yeast

Inducibility of AtUPS1 synthesis after galactose induction provides a useful tool for monitoring plasma membrane delivery, as previously demonstrated for the yeast Fur4p [27]. We measured uracil uptake activity after galactose induction of GAL-*UPS1* and GAL-*FUR4* in *fur4Δ* cells in parallel (Fig. 2A). In both cases, activity, as a measure of plasma membrane targeting, was detectable after 30 min induction. The increase in uracil uptake activity was linear for one hour with a similar slope for cells expressing the yeast and the plant transporters. Hence the heterologous AtUPS1 appears to be recognized efficiently by the yeast secretory machinery. We then compared the fate of AtUPS1 and Fur4p along the endocytic pathway using an experimental condition known to trigger rapid Fur4p internalization and subsequent vacuolar degradation, *i. e.* the inhibition of protein synthesis by addition of cycloheximide [28] (Fig. 2B). Cycloheximide was added to exponentially growing *fur4Δ* cells induced overnight in galactose for expression of either AtUPS1 or Fur4p.

Uracil uptake activity of cells expressing Fur4p decreased rapidly ($t_{1/2}$= 45 min), whereas uracil uptake activity of cells expressing AtUPS1 did not display a decrease for four hours. Hence, once delivered to the plasma membrane, the plant AtUPS1 was remarkably stable, and did not undergo obvious endocytosis after inhibition of protein synthesis.

C-terminally tagged versions of AtUPS1 are retained in the ER

The monitoring of uracil uptake activity can afford insight into the intracellular fate of AtUPS1, since it provides information about the plasma membrane located transporter. This information, however, remains limited, and does not indicate the fraction of plasma membrane-delivered protein versus potential intracellular pools. In the absence of available antibodies, we decided to monitor the fate of tagged versions of AtUPS1. C-terminally tagged versions of AtUPS1 were constructed, first with a GFP-tag, a powerful tool for monitoring the intracellular fate in yeast of plasma membrane proteins of heterologous [14] or endogenous origin, including that of Fur4p [29-31]. *fur4Δ* cells transformed with a multicopy plasmid carrying GAL-*UPS1-GFP* displayed high 5FU sensitivity after growth on galactose (Fig. 3A). Strikingly, *fur4Δ* cells transformed with multicopy plasmid carrying GAL-*UPS1* tagged with the smaller HA epitope displayed intermediate 5FU sensitivity after growth on galactose, indicating that a smaller tag did not improve AtUPS1 functionality. According to these plate assays, C-terminally tagged AtUPS1 thus appeared functional, notably GFP-tagged AtUPS1. However, *fur4Δ* cells transformed by the multicopy plasmid carrying GAL-*UPS1-GFP* and fully induced displayed only a very low level of uracil uptake activity (0.04 nMol/min/A_{600}), an activity 50 fold lower than that observed in the case of induced cells transformed by a plasmid carrying untagged AtUPS1 (not shown).

The use of a smaller tag (HA) or another promoter (CYC1) did not improve uracil uptake activity.

The GFP -or HA- tags may inhibit transport activity or impair plasma membrane delivery of tagged transporter due to folding problems. We checked GFP fluorescence in time course experiments using the multicopy plasmid carrying the GAL-*UPS1-GFP* (Fig. 3B), or the multicopy plasmid GAL-*Fur4-GFP* as a control. Galactose induction of Fur4-GFP led, after 30 min, to observation of small internal compartments, likely Golgi/secretory vesicles. 30 min later, plasma membrane staining was clearly evidenced, often in a polarized fashion, with intense staining of small buds (Fig. 3B). The distribution of AtUPS1-GFP was strikingly different. After 30 min of galactose induction of AtUPS1-GFP expression, a perinuclear staining was clearly observable. 30 min later, or after overnight induction, the perinuclear staining was still present and a discontinuous staining at/or underneath the plasma membrane has appeared. This pattern is typical of the yeast ER. Intense staining of lines or spots, as if intracellular membranes had formed aggregates were also visualized (Fig. 3B). Indeed, electron microscopy of the ultrastructural morphology of cells fully induced for expression of AtUPS1-GFP revealed proliferation, hanks of ER membranes (Fig. 3D), as often described in the case of cells overexpressing ER-retained proteins [11]. This altered morphology did not resulted from the overexpression of a membrane protein as such: overnight overexpression of endogenous *FUR4* from the same multicopy, GAL-inducible plasmid, did not lead to altered morphology (not shown).

ER-retention of GFP-tagged AtUPS1 apparently did not result from the mere overexpression of this protein. Perinuclear/ER staining was also evidenced upon expression of AtUPS1-GFP from a centromeric plasmid under the control of the mild

strength *CYC1* promoter, leading to a steady state protein level about 4-fold lower than that observed after two hours galactose induction of GAL-*UPS1-GFP* (Fig. 3B and C). Furthermore, when analyzed by sucrose gradient fractionation, AtUPS1-GFP expressed from the CEN CYC1-plasmid or from the 2 μ GAL-inducible plasmid displayed exactly the same pattern with major pool in internal fractions (data not shown).

Hence, the GFP tag at the C-terminus of AtUPS1 obviously triggers ER retention of the transporter. The low uracil uptake activity of cells expressing this transporter can only be observed after long expression periods (3-4 hours, cf Fig. 6B), and likely corresponds to the low fraction of protein finally reaching the plasma membrane. With such folding problems, overexpression is a way to increase steady state plasma membrane expression: both 5FU sensitivity and uracil uptake activity were improved in the case of AtUPS1-GFP expression from a multicopy plasmid and strong GAL promoter compared to expression from a centromeric plasmid under the control of a lower strength promoter.

Intracellular fate of AtUPS1 carrying an internal myc tag in yeast: slow ER exit but final plasma membrane delivery

We checked whether insertion of a small myc-tag inside the central loop containing the Walker A motif would be a better way to study AtUPS1 intracellular trafficking. Cells expressing internally myc tagged AtUPS1 from a multicopy galactose inducible plasmid displayed high sensitivity to 5FU (Fig. 4A). Cells transformed with the multicopy GAL-*UPS1*^{myc} plasmid grown overnight in galactose displayed a relatively high level of uracil uptake activity: 1 nMol/min/A_{600}, *i. e.* 70% that observed in the case of untagged AtUPS1 (Fig. 4B), suggesting that the myc tag

at this position had only a small impact on AtUPS1 function. However, this tag had a clear influence on AtUPS1 intracellular trafficking. Induction experiments showed that uracil uptake activity appeared slowly: 4-6 hours were necessary before we were able to measure any detectable uptake activity (Fig. 4C). Aliquots withdrawn at several time points after galactose induction were analyzed by protein gel blots using a specific anti-myc antibody. AtUPS1^{myc} appeared on gels as a band of apparent molecular mass of about 36 kDa, *i. e.* slightly below the expected molecular mass deduced from predicted protein sequence (44 kDa) (Fig. 4E), as often observed for very hydrophobic proteins. A comparable pattern was observed after overnight galactose induction of AtUPS1^{myc} expressed from either a centromeric or a multicopy plasmid (data not shown).

In order to check the intracellular location of AtUPS1^{myc} derived species, cells transformed with GAL-*UPS1*^{myc} were induced by addition of galactose, aliquots withdrawn periodically were fixed and analyzed by immunofluorescence using a monoclonal anti-myc antibody. One hour induction was sufficient to observe a specific signal, mostly perinuclear, supported by simultaneous DAPI staining (Fig. 5A). To obtain better resolution of the cell surface staining (potentially corresponding to either ER or plasma membrane), cells were examined by confocal microscopy. The lower background in optical slices made it possible to show that all cells displayed perinuclear staining, together with discontinuous regions of cell surface staining (Fig. 5B). Because cell surface was not homogeneously stained, we can conclude that the main signal corresponds to ER staining and that a plasma membrane localisation cannot be detected by this approach. The low rate of uracil uptake activity thus likely resulted from low ER exit rates (Fig. 4C) The finding that the activity of fully induced cells is in a similar range as that of cells expressing

untagged AtUPS1, which was rapidly targeted to plasma membrane, may suggest that after slow rates of ER exit, AtUPS1^{myc} finally reached the plasma membrane. Further indication that AtUPS1^{myc} stored in internal compartments could finally reach plasma membrane was provided by the observation of some increase in uracil uptake activity after stopping transporter synthesis by the addition of CHX (Fig. 4D). Once targeted at the plasma membrane, AtUPS1^{myc} was rather stable, as judged from the extreme stability of uracil uptake activity for over 3 hours after this transient uracil uptake activity increase following CHX addition. However, some low rate endocytosis likely occurred as compared to the incredibly stable untagged AtUPS1 (Fig. 4D).

Mutations in the Rsp5 ubiquitin protein ligase improve functional expression of tagged and untagged AtUPS1 in yeast

Despite the difficulties encountered in tagging AtUPS1 at different positions, the high sensitivity of the two functional tests, 5FU sensitivity and uracil uptake measurements, provided suitable tools to check whether yeast mutants could improve the steady state levels of functional tagged or untagged AtUPS1 versions. We used two viable *rsp5* mutants, *npi1* and *rsp5ΔC2,* that display delayed endocytosis of several cargoes, including Fur4p. Accordingly, they displayed increased 5FU sensitivity as a result of the plasma membrane stabilization of endogenous chromosomal or plasmid encoded Fur4p (not shown**).** We checked the fate of tagged and untagged versions of AtUPS1 in *npi1* and *rsp5ΔC2* mutant cells.

For this purpose, *FUR4* was deleted in wild type, *npi1* and *rsp5ΔC2* cells, and the resulting strains were transformed with multicopy plasmids carrying either *UPS1*

under the control of the constitutive PGK promoter (plasmid pFL61-*UPS1*), or galactose inducible *UPS1, UPS1-GFP and UPS1*^{myc}. Transformed cells grown in glucose, or galactose containing media in the case of plasmids with a GAL promoter displayed identical growth in the absence of 5FU (Fig. 6A), indicating that neither the mutations, nor the expression of the plant transporters impaired growth. 5FU sensitivity of transformants was strikingly enhanced in both *rsp5ΔC2* and *npi1* cells expressing the tagged and untagged plant transporter when compared to wild type cells, with slightly stronger effect promoted in all cases by the *npi1* mutation (compare the size of isolated colonies) (Fig. 6A). This suggests higher amounts of plasma membrane tagged or untagged transporters in the mutants. In the case of the untagged transporters, the enhancement in 5FU sensitivity was evidenced after synthesis from PGK or *GAL10* promoter at different 5FU concentrations (Fig. 6A), indicating that *rsp5* mutations interfered with trafficking, rather than with galactose-driven expression. For untagged AtUPS1 which is rapidly delivered to the plasma membrane and very stable in wild type cells, the increase in functional transporter activity observed in *rsp5* αmutants may result from inhibition of some direct Golgi to vacuole targeting, or from protection against a possible low basal endocytosis, for instance when cells reached stationary phase, a likely situation for cells grown several days on plates.

In the case of the myc-tagged version of AtUPS1, we monitored the fate of the transporter after galactose induction by western blots (Fig 6C), and uracil uptake measurements (Fig. 6B). In agreement with fluorescent data, AtUPS1^{myc} protein was already detectable in wild type cells at early time points after induction (30-60 min) but uracil uptake was readily measurable only at far later time points (4 hours). In *npi1* cells, AtUPS1^{myc} was detectable in higher amounts at all time points and uracil

uptake activity appeared at least one hour earlier compared to wild type cells (Fig. 6B). *rsp5ΔC2* mutation also resulted in more rapid appearance of uracil uptake activity. Strikingly, even if the activity was lower for cells expressing AtUPS1-GFP compared to AtUPS1^{myc}, the rate of appearance of uptake was very similar. For both types of tagged transporters uracil uptake activity was thus 2-3 fold higher in mutant cells than in wild type cells after 4 hour induction. Since the major pool of both AtUPS1-GFP and AtUPS1^{myc} seemed to be in the ER, both *npi1* and *rsp5ΔC2* mutations apparently lead to accelerated ER exit.

Discussion:

The use of a regulable promoter and the high sensitivity of uracil uptake measurements enabled us to obtain crucial information about the fate of untagged AtUPS1 uracil transporter in yeast. AtUPS1 is rapidly delivered to the plasma membrane, with kinetics indistinguishable from those of endogenous yeast transporter. Hence, the yeast secretory pathway efficiently handles the heterologous plant transporter. It is possible that such property was the reason for the successful cloning by functional expression of so many plant transporters when using yeast as a host system [6,7]. Plasma membrane delivered AtUPS1 exhibited striking stability, notably when compared to the endogenous yeast uracil permease especially susceptible to stress-induced endocytosis [28]. This was true for both untagged transporter and for internally tagged AtUPS1^{myc} (not shown). This differential stability between yeast and plant uracil transporters may possibly reflect fundamental differences in their endocytic processes. Endocytosis in yeast is dependent on prior ubiquitylation of plasma membrane cargoes by the Rsp5p ubiquitin protein ligase [17]. A subset of mammalian proteins undergo ubiquitin-dependent endocytosis,

sometimes involving ubiquitin-protein ligases of the Nedd4/Rsp5 family [32]. In contrast, although plants display numerous ubiquitin protein ligases, they do not seem to have Rsp5p orthologs [33]. The extreme stability of AtUPS1 in yeast could result from the absence or low accessibility of ubiquitylation sites that Rsp5p can recognize, at least under the experimental conditions we tested. One possible reason could be the lumenal orientation of both AtUPS1 N- and C-termini.

In addition to obtaining information on intracellular trafficking of the plant AtUPS1 in yeast based on uptake measurements, we tried to gain new insights into the biochemical properties of this transporter. Unfortunately, tagging the transporter, either at the N-terminus (not shown) and C-terminus or inside its central loop, lead to folding problems, often resulting in ER retention of most of the protein, preventing us from using these tagged versions to obtain a judicious biochemical characterization of this transporter. A marked difference, however, was observed between internal tagging, and tagging at the N- and C-termini, which was far more deleterious. Internally tagged transporter displayed delayed ER exit, but finally reached the plasma membrane in a fully functional state, and fully induced cells expressing this version of the transporter displayed activity closely resembling that of cells expressing untagged transporter. In contrast, cells expressing C-terminally tagged transporter displayed 40-fold less uracil uptake activity compared to cells expressing untagged transporters, probably because of a very low percentage of the protein exiting the ER. In latter case, some improvement could be achieved by increased levels of expression (stronger promoter, multicopy versus centromeric plasmid). The deleterious effect of C-terminal tags could result from the unusual structure of the transporter. Hydrophobicity plot analysis of proteins of the UPS family suggested an external orientation of both termini [15] a situation somewhat rare for transporters.

Most yeast transporters, for instance, display cytoplasmic oriented termini, as predicted from hydrophobicity plot analysis or from experimental data [2]. Many of these tranporters were studied after tagging, most often at their C-terminus, a modification that did not induce any major trafficking problems. The observation that N- and C-terminal tags impaired AtUPS1 folding is compatible with an external orientation, but experimental data are required to prove this. One obvious lesson from these observations is that tagging at the positions and with the tags used in the present study may probably influence the fate of UPS proteins in plant cells in a similar way as in yeast cells. Indeed, previous studies attempting to study the subcellular location of N- or C-terminal fusions of UPS proteins with GFP by transient expression in Arabidopsis protoplasts showed that these fusion proteins did not reach the plasma membrane (Schmidt, A., Baumann, N. and Desimone, M., unpublished data). This suggests that yeast may also represent a useful experimental system to decipher where tags can be introduced in heterologous transporters.

The distinctive behaviour of the various versions of AtUPS1, one correctly targeted to the plasma membrane, and two retained in the ER to various extents, provided the opportunity to check how to improve the steady state plasma membrane amount of heterologous transporters with distinct intracellular fates. We checked specifically the influence of mutations in Rsp5p. This ubiquitin protein ligase is involved in various stages of intracellular trafficking of membrane-bound proteins, including ER-associated degradation, plasma membrane internalization, Golgi to vacuolar trafficking, and sorting to multivesicular bodies [17]. Limiting or preventing latter three trafficking steps results in elevated steady state levels of yeast transporters. Two specific viable mutants were used, *npi1*, with decreased amounts

of Rsp5p [25] and *rsp5ΔC2*, lacking the C2 domain of Rsp5p [34], dispensable for viability, but critical for Rsp5p trafficking functions [26,34-38]. Strikingly, both mutants improved steady state amounts of functional, plasma membrane targeted, tagged and untagged versions of AtUPS1, as judged from increased 5FU sensitivity. In the case of untagged transporter, increased 5FU sensitivity was observed after expression of the transporter under the control of the constitutive PGK promoter, or the inducible *GAL10* promoter, indicating that the mutations interfered as expected with trafficking rather than with expression.

In the case of the untagged version of AtUPS1, it was difficult to further explore the mechanism of this improvement. Plasma membrane AtUPS1 was so stable in wild type cells submitted to CHX treatment that further stabilisation in *rsp5* cells could not be detected in this type of experiment. The improvement in plasma membrane steady state amounts of AtUPS1 in *npi1* and *rsp5ΔC2* cells might possibly arise from a low basal endocytosis of AtUPS1, undetectable in CHX chase experiments, but possibly occurring once cells reach stationary phase, as is the case for cells grown on plates. Alternatively, the increase in plasma membrane AtUPS1 in these *rsp5* mutants could result from a decreased direct Golgi to vacuole targeting of a fraction of the transporter.

Mutations in *RSP5,* either *npi1* or *rsp5ΔC2,* had a similar impact on the intracellular fate of tagged versions of AtUPS1 at both the C-terminus or in the intracellular loop. These two variants of AtUPS1 were mainly located in the ER, with possibly exit in only very limited amounts (AtUPS1-GFP), or more important final amounts (AtUPS1^{myc}) thus leading to uracil uptake activity similar to that displayed by cells expressing untagged transporters. In both cases, the rate of plasma membrane delivery of uracil uptake activity was greatly delayed, with activity measurable after 3-

4 hours induction instead of 30 min for AtUPS1 –i. e. long after detection of the protein on Western blots- and the two *rsp5* mutations reduced this delay. At first glance, this effect can be attributed to an effect of Rsp5p on ER exit. Among its many functions, Rsp5p was described to be involved in ubiquitylation followed by proteasome degradation of several misfolded soluble and membrane-bound proteins retained in the ER, notably under conditions of saturation of other ER-associated degradative (ERAD) pathways [39]. Tagged forms of AtUPS1 might be partly susceptible to such an Rsp5p-dependent ER-associated degradation, and inhibition of this degradation in *npi1* and *rsp5ΔC2* mutants would result in more rapid ER exit of transporters escaping this degradation to some extent. It is also possible that a small number of misfolded tagged transporters undergo ER to Golgi trafficking, followed by Golgi to vacuole sorting. Rsp5p was also shown to be involved in Golgi to vacuole trafficking of some misfolded mutant plasma membrane proteins [22] or of yeast transporters displaying direct vacuolar targeting under specific nutrient conditions [17]. In both cases in *rsp5* mutants, these proteins are directed to plasma membrane [22,40]. A small fraction of tagged AtUPS1 could undergo such direct vacuolar trafficking, a process inhibited in *rsp5* mutants. In support of a partial direct Golgi to vacuole trafficking of AtUPS1^{myc}, we observed that *pep4* mutant cells, deficient for vacuolar protease activities, displayed higher amounts of the 36 kDa AtUPS1^{myc} species than wild type cells even after short induction times, as did *vps23Δ* mutants, impaired in Golgi to vacuole trafficking (data not shown). The increase in plasma membrane amounts of functional forms of tagged AtUPS1 in *rsp5* mutants, attested in exponentially growing cells by increased uracil uptake activity, and on plates by increased 5FU sensitivity might also result from an inhibition of Rsp5p-dependent

processes at three levels: ERAD, Golgi-to-vacuole trafficking and plasma membrane internalization.

Rsp5p plays a key role in trafficking of yeast plasma membrane proteins. The present study shows that several viable *rsp5* mutants display increased plasma membrane amounts of a plant transporter, rapidly targeted to the plasma membrane. Strikingly, within the same european network (EFFEXPORT), other laboratories observed, in viable *rsp5* mutants, improved functional expression of several heterologous transporters, including several $NH4^+$ transporters (Rh family) of animal origin (Marini A and André B, personal communication), and increased plasma membrane amounts versus internal fractions of mammalian Na^+/H^+ antiporters (Flegegova, H, Haguenauer-Tsapis, R and Sychrova, H. Biochem. Biophys. Acta, in press). In addition, these mutants optimize plasma membrane delivery of tagged versions of the plant AtUPS1, stacked in the ER as a result of folding problems. ER retention is one of the major problems encountered in the case of expression of heterologous plasma membrane proteins in yeast. The increase in steady state plasma membrane amounts of heterologous plant and mammalian transporters, upon expression in viable *rsp5* mutants, show that these cells could be powerful tools to solve the many problems inherent in heterologous expression of membrane proteins, including ER retention.

Materials and methods

Yeast strains and growth conditions

Yeast strains were transformed as described by Gietz et al [41]. Cells were grown at 30°C in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino

acids (BD bioscience, NJ, USA), and supplemented with appropriate nutrients. The carbon source was 2% glucose, or 2% galactose plus 0.05% glucose as indicated in the figure legends. Galactose induction was performed on cells grown overnight in 2% raffinose plus 0.05% glucose up to an A_{600nm}=0.5. Galactose (2%) was then added to the medium.

The disruption of *FUR4* gene was achieved by ORF replacement with long flanking homology regions to the KanMX4 cassette corresponding to the strategy described by Wach [42].

Growth tests in the presence of 5-fluorouracil

Cells, prototroph for uracil, were cultured overnight in minimal medium containing glucose and spotted on plates containing minimal medium with galactose to induce expression of *FUR4* or *UPS1* variants and supplemented with various concentrations of 5FU (Sigma-Aldrich, Lyon, France). The first drop contained 3.10^4 cells and each subsequent drop was diluted six-fold compared to the prior drop.

Construction of plasmids

DNA manipulations, including restriction analysis and ligations, were performed essentially as described by Maniatis *et al.* [43].

The control plasmid p195gF-GFP (pRT208) expressing FUR4-GFP under the control of the GAL10 promoter was constructed by cloning a PstI/BamH1 fragment (FUR4-GFP) from pFL38gF-GFP [30] into p195gF [28] at the PstI/BamHI site.

The pGAL-*UPS1* (pRT205) plasmid was constructed by insertion of a BamHI/EcoRI fragment encoding *UPS1* (with pFL61-*UPS1* as a template [15]) in the BamHI/EcoRI site of pYEF1 [44].

To construct the plasmid GAL-*UPS1-GFP* (pRT206) we first built the pNBT29 plasmid, which contains the yeast enhanced GFP under the control of the GAL10 promoter on a multicopy plasmid. For this purpose, a BamHI/NotI fragment encoding GFP was obtained by PCR using the pUG35 plasmid as a template [45] and introduced at the BamHI/NotI site of pYEF1 [44]. Then a BamHI/ClaI fragment encoding AtUPS1 was amplified by PCR using pFL61-*AtUPS1* [15] as a template, and introduced at the BamHI/ClaI site of pNBT29 in frame with the coding sequence of GFP thereby creating a GFP C-terminally tagged version of AtUPS1. To construct the CYC1-*UPS1-GFP* (pRT207) plasmid we cloned the BamHI/EcoRI fragment containing *UPS1-GFP* at the BamHI/EcoRI site of the p416-CYC1 plasmid [46] **.**

The pGAL-*UPS1-HA* (pRT204) plasmid was obtained by insertion in the ClaI site of the pYEF2 [44]**,** in frame with the coding sequence of the HA tag, of a fragment encoding *UPS1* obtained by restriction of the plasmid GAL-*UPS1-GFP*.

To construct the plasmid pGAL-*UPS1*^{myc} (pRT203) two fragments of *UPS1* were amplified separately by PCR using pFL61-*UPS1* as a template. One fragment contained at start an EcoRI and an XbaI site, the 5'- portion of the *UPS1* coding sequence (from ATG to the position 549) and a BamHI site at the end. The other fragment contained a BamHI site at the start, the coding sequence for the c-myc epitope, the 3'- portion of *UPS1* (from position 550 to the stop codon), and an EcoRI and a XhoI site at the end. Both fragments were sequentially cloned in pDR199 [47] using the EcoRI/BamHI sites for the first fragment and BamHI/and XhoI for the second. After sequencing, the complete c-myc tagged *UPS1* sequence was obtained by restriction and subcloned into the Xba I / Xho I sites of the CEN plasmid p416-GAL [46]. Afterwards, the multicopy plasmid pGAL-*UPS1*^{myc} was obtained by

subcloning a SacI/XhoI fragment of the p416-GAL-*UPS1*^{myc} into the SacI/XhoI sites of the p426-GAL plasmid [46].

Measurement of uracil uptake.

Uracil uptake was measured in exponentially growing cells as previously described. Yeast culture (1 ml) was incubated with 5 µM [^{14}C] uracil (ICN biomedicals Illkirch, France) for 20 sec at 30°C, then quickly filtered through Whatman GF/C filters, which were in turn washed twice with ice-cold water and counted for radioactivity. In the case of low uracil uptake activity, this basic protocol was slightly modified, with the use of two ml samples and incubation for 2 min at 30°C.

Michaelis-Menten kinetics

Uracil uptake activities measured at various substrate concentrations were fitted to a hyperbola with SIGMA PLOT 5.0, V5 according to Michaelis-Menten kinetics.

Yeast cell extracts, SDS-PAGE and Western immunoblotting

Total protein extracts were prepared by the NaOH/Trichloroacetic acid (TCA) lysis technique as described in [28]. Proteins were separated by SDS-PAGE on Tricine gels and transferred onto nitrocellulose membranes. The membranes were probed with monoclonal antibodies against GFP (Roche Diagnostics Meylan, France), or myc (9E10 from Roche Diagnostics), or polyclonal antibody against Gas1p (a kind gift from H. Riezman). Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Sigma-Aldrich, Lyon, France) revealed by ECL chemiluminescence (Amersham).

Immunofluorescence

Immunofluorescence was performed as described in [48] except that cells were permeabilized with 0.5% Triton X100. The primary antibody was the monoclonal anti-Myc (9E10 from Roche Diagnostics, Meylan, France)and the secondary antibody was an FITC-conjugated goat anti–mouse-IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For DNA staining, 1 μg/ml Diamin-Phenylindol-Dihydrochlorid (DAPI) was used. Samples were viewed under an Olympus microscope BY61 using FITC and DAPI filter sets. Image acquisition was performed using a Spot charge-coupled device camera SPOT4.05 .

For confocal analysis, cells were imaged using an inverted microscope (Leica, Inc. Wetzlar, Germany) and scanning was performed with a True Confocal Scanner LEICA TCS 4D.

Electron microscopy

Yeast cells were fixed by adding 200 µl of 50% aqueous glutaraldehyde to 10 ml of growth medium for 10 min and then centrifuged at 5000 g for 10 min at 4°C. After fixation with fresh fixatives for 2 h at 4°C, cells were washed in 0.1 M cacodylate buffer (pH 7.4) and in water. Subsequently, cells were treated with 1% KMnO4 for 2 h on ice, washed in water and re-suspended in 2% aqueous uranyl acetate for 1 h at 4°C. Cells were dehydrated in a graded series of ethanol, infiltrated in a mixture of ethanol and Spurr's resin and embedded in Spurr's low viscosity media. Thin sections were cut, stained with lead citrate and examined in a Tecnai 12 electron microscope (Eindhoven, Netherlands).

TABLE 1. List of strains

Strain	Background	Genotype	Source
MF04	Σ1278b	*MAT**a** ura3 trp1 FUR4::KanMX4*	This study
MF05	Σ1278b	*MAT**a** ura3 trp1 rsp5ΔC2 FUR4::kanMX4*	This study
MF06	Σ1278b	Mat**a** *ura3 trp1 npi1 FUR4::kanMX4*	This study
27061b	Σ1278b	Mat**a** *ura3 trp1*	[19]
27064b	Σ1278b	Mat**a** *ura3 trp1 npi1*	[19]
BY4741	BY	Mat**a** *leu2Δ met15Δ ura3Δ his3Δ*	Euroscarf

TABLE 2. List of plasmids

Plasmid	Characteristics	Source
pPGK-*UPS1* (PFL61-*UPS1*)	2μ, *URA3* prom. *PGK-UPS1*	[15]
pGAL (PYeF2)	2μ, *URA3*, prom. *GAL10, Cter HA*	[44]
pGAL-*FUR4* (pFL38gF)	CEN, *URA3,* prom. *GAL10, FUR4*	[30]
p195gF	2μ, *URA3*, prom. *GAL10, FUR4*	[28]
p195gF-GFP (pRT208)	2μ, *URA3*, prom. *GAL10-FUR4-GFP*	This study
pGAL-*UPS1-HA* (pRT204)	2μ, *URA3*, prom. *GAL10, UPS1- HA*	This study
pGAL-*UPS1* (pRT205)	2μ, *URA3,* prom. *GAL10*, *UPS1*	This study
pGAL-*UPS1-GFP* (pRT206)	2μ, *URA3,* prom. *GAL10, UPS1-GFP*	This study
pCYC1-*UPS1-GFP* (pRT207)	CEN, *URA3*, prom.*CYC1, UPS1-GFP*	This study
pGAL-*UPS1*^{myc}*(pRT203)*	2μ, *URA3,* prom. *GAL10, UPS1-myc*	This study

Figure 1: **Comparison of uracil uptake activity of *Arabidopsis thaliana* UPS1 expressed in yeast and endogenous uracil permease Fur4p.**

A: 5-Fluorouracil (5FU) sensitivity. *fur4*Δ cells transformed with either pGAL (empty vector), pGAL-*UPS1* or pGAL-*FUR4* were grown on galactose containing plates supplemented or not with 1μM 5FU (toxic analog of uracil).

B: Uracil uptake activity of AtUPS1 compared to Fur4p. *fur4*Δ strains transformed with pGAL (white), pGAL-*UPS1*(grey) or pGAL-*FUR4 (*black) were grown to exponential phase in galactose containing medium and used for measurement of [^{14}C] uracil uptakeas described in Materials and Methods. Results are the average of four measures (two measures in two independent experiments).

Figure 2: **Intracellular trafficking of AtUPS1 in yeast.**

A: *fur4*Δ strains transformed with pGAL-*UPS1* (triangle) or pGAL-*FUR4* (circle) were grown with raffinose as a carbon source. Galactose was then added to induce expression of *AtUPS1* and *FUR4.* The kinetics of plasma membrane delivery of AtUPS1 and Fur4p was determined by quantification of [^{14}C] uracil uptake every 30 minutes after galactose induction. Results at each time point are the average of two independent measurements.

B: *fur4*Δ strains transformed with pGAL-*UPS1* (triangle) or pGAL-*FUR4* (circle) were grown in galactose containing medium. Protein synthesis was inhibited by addition of cycloheximide (CHX) (100μg/ml). Uracil uptake activity was measured at various time points (two measurements) after CHX addition. Results are shown as the percentage of initial activities.

Figure 3: **Expression of C-terminally GFP tagged AtUPS1 promotes ER proliferation in yeast**

A: *fur4*Δ cells transformed with pGAL, pGAL-*UPS1-HA,* pGAL-*UPS1-GFP and pCYC1-UPS1-GFP* were tested for growth on plates containing or not 5-fluorouracil (0,75 μM).

B and C: *fur4*Δ cells transformed with pCYC-*UPS1-GFP,* pGAL-*UPS1-GFP* or pGAL-*Fur4-GFP* were grown to mid exponential phase either in glucose, or in raffinose containing medium in the case of strains bearing a plasmid with a GAL promoter. Galactose was then added to induce *AtUPS1-GFP or Fur4-GFP* expression. At the indicated times, after galactose addition or after growth in glucose containing media (in the case of pCYC-*UPS1-GFP*), cells were observed by fluorescence microscopy (B) and protein extracts were prepared, resolved by SDS PAGE and analysed by Western immunoblotting using an anti-GFP antibody (C)

D: *fur4*Δ cells expressing pGAL and pGAL-*UPS1-GFP* grown overnight in galactose containing media were processed for electron microscopy. White arrows indicate the endoplasmic reticulum and N the nucleus.

Figure 4: **Intracellular fate of myc tagged version of AtUPS1**

A: *fur4*Δ cells transformed with pGAL, pGAL-*UPS1*, and pGAL-*UPS1*^{myc} were tested for 5-fluorouracil (1μM) sensitivity on plates.

B: [^{14}C] uracil uptake activity of *fur4*Δ cells transformed with pGAL-*FUR4*, pGAL-*UPS1* and pGAL-*UPS1*^{myc} grown to mid exponential phase on galactose containing medium. Results are the average of two independent measures.

C: *fur4*Δ strains transformed with pGAL-*UPS1* (triangle) or pGAL-*UPS1*^{myc} (square) were grown to mid log phase in raffinose containing medium and galactose was then

added to induce expression of the transporter. The kinetics of plasma membrane delivery of AtUPS1 or Fur4p were determined by quantification of [^{14}C] uracil uptake at various times after galactose induction.

D: *fur4*Δ strains transformed with pGAL-*UPS1* (triangle) or pGAL-*UPS1*^{myc} (square) were grown in galactose containing medium. Uracil uptake activity was measured at different time points after inhibition of protein synthesis by addition of CHX (100μg/ml). Results are shown as the percentage of initial activity.

E: WT cells transformed with pGAL-*UPS1*^{myc} were grown to mid log phase in raffinose containing medium. Galactose was then added to induce transporter expression. Protein extracts were prepared at indicated times and proteins were resolved by SDS PAGE and analysed by Western immunoblotting using an anti-myc antibody to detect the transporter and an anti-Gas1p as a loading control.

Figure 5: **Intracellular localization of UPS1^{myc} after galactose induction.**

A: *fur4*Δ cells transformed with pGAL-*UPS1*^{myc} were grown overnight in raffinose containing media. Galactose was added during exponential growth phase. Aliquots were withdrawn at various time points, cells were fixed and processed for immunofluorescence. AtUPS1^{myc} was detected using an anti-myc antibody as described in Materials and Methods. The nuclei were stained using DAPI. Fluorescence was observed under an Olympus microscope.

(Note that, as it is often the case for multicopy plasmid and galactose induction, only a subset of the cells within the population was stained with the anti-myc antibody)

B: The same preparations as in (A) were also visualized using confocal microscopy. One section is presented for each time point.

Figure 6: **Mutations in *RSP5* improve functional expression in yeast of tagged and untagged UPS1**

A: *fur4*Δ*, rsp5*Δ*C2 fur4*Δ and *npi1 fur4*Δ cells transformed with pPGK-*UPS1*, pGAL-*UPS1*, pGAL-*UPS1-GFP* or pGAL-*UPS1*^{myc} were grown in glucose containing medium and spotted either on glucose (pPGK-*UPS1*) or on galactose containing plates (pGAL plasmids) with or without5-fluorouracil as indicated. Different 5FU concentrations were used in order to differentiate optimally the growth of each of the tested strains with regard to the various plasmids: concentration was lower in the case of galactose-induced cells expressing untagged and myc-tagged AtUPS1 that display greater uracil uptake activities than cells expressing GFP-tagged AtUPS1, or untagged AtUPS1 under the control of the PGK promoter. In all cases, growth was tested using a whole range of 5FU concentrations, and a selection of representative plates is shown.

B: *fur4*Δ (triangle), *rsp5*Δ*C2 fur4*Δ (square) and *npi1 fur4*Δ (diamond) transformed with pGAL-*UPS1-GFP* or pGAL-*UPS1*^{myc} were grown to mid log phase in raffinose containing media and the expression of the various transporters was induced by addition of galactose. Plasma membrane delivery of the transporters was assessed by measuring uracil uptake activity at different time points after galactose addition.

C: Induction of AtUPS1^{myc} was monitored in *WT* and *npi1* cells by Western blot analysis of aliquots withdrawn at several time points after addition of galactose. Proteins were resolved by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-myc antibodies for quantification of transporter expression and anti-Gas1p as loading control.

Acknowledgements

We thank H. Riezman for the kind gift of antibodies, Antonia Kropfinger for editorial assistance, and the members of RHT's laboratory for helpful discussions and comments on the manuscript and. We are grateful to Sophie Le Panse for electron microscopy. We also thank Jennifer Molinari, Youri Lokossa, Ludovic Warroux and Yvonne Sauermann for technical help. This work was supported by a grant to M.D. from the Deutsche Forschungsgemeinschaft (DE 817/1-1).The work done in RHT's lab was supported by the Centre National de la Recherche Scientifique, the Universities Paris 6 and Paris 7, by a grant from the Association pour la Recherche contre le Cancer (ARC) (grant no. 3298), and by an European Union program (EFFEXPORT, contract QLRT-2001-00533). Marine Froissard, Naïma Belgareh-Touzé and Jennifer Molinari received fellowships from this program. We are especially indebted to Bruno Andre, coordinator of the EFFEXPORT program, for his constant support.

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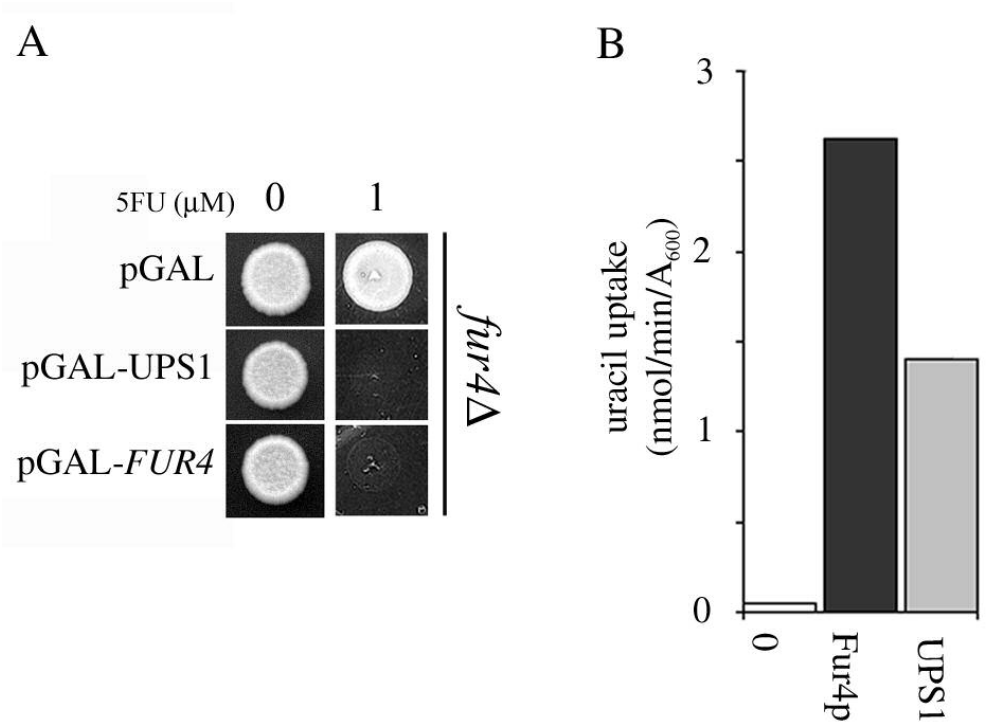
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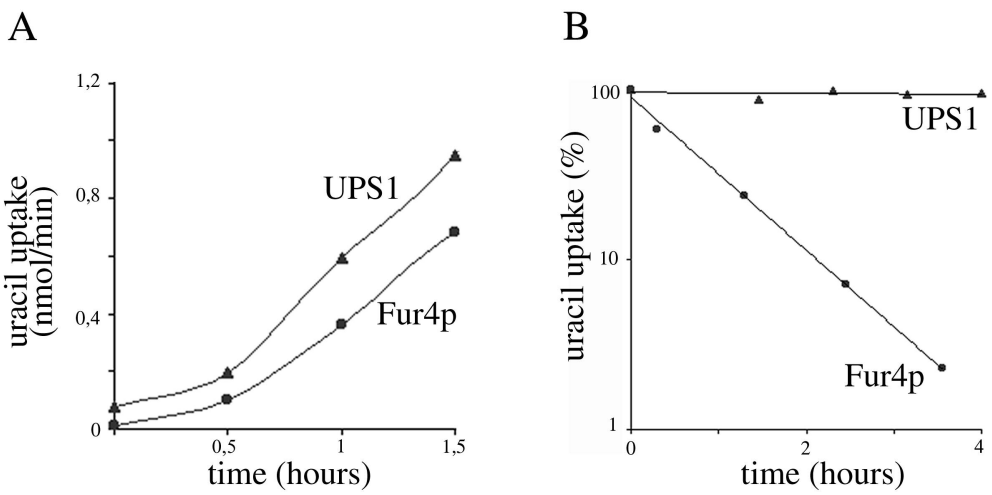
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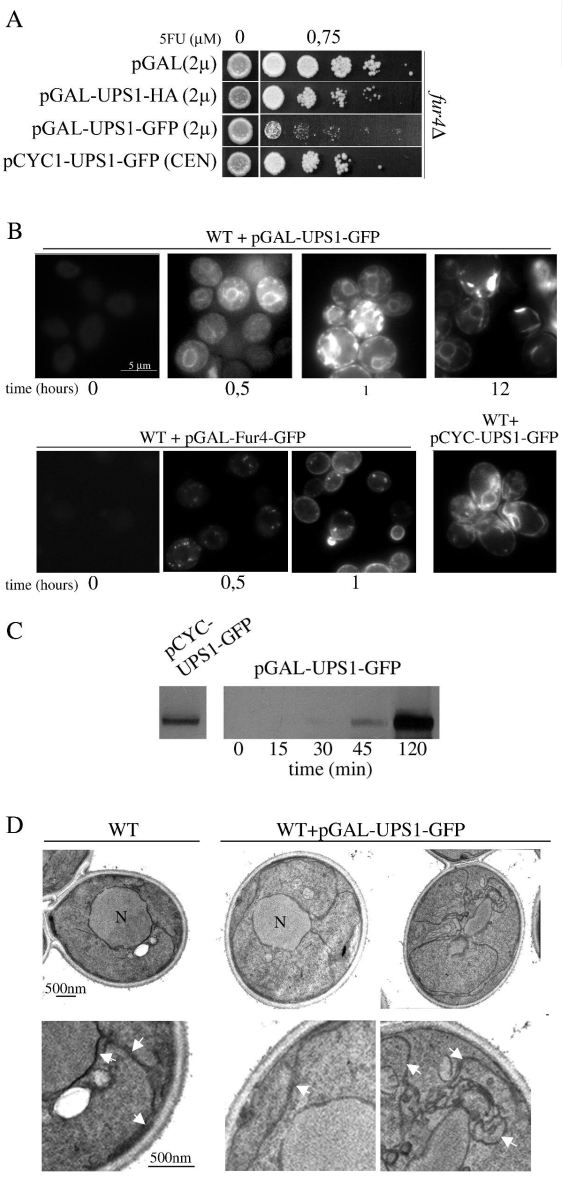
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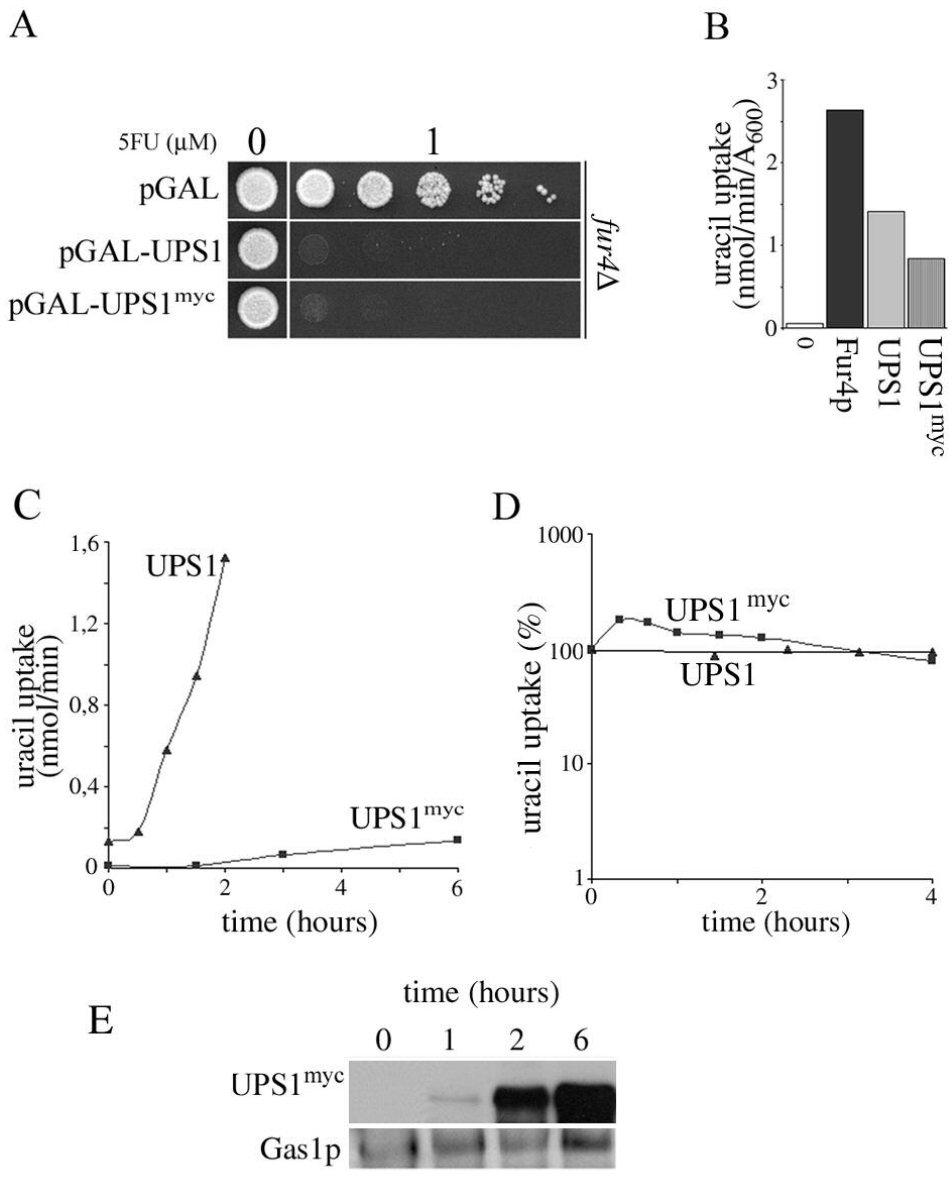
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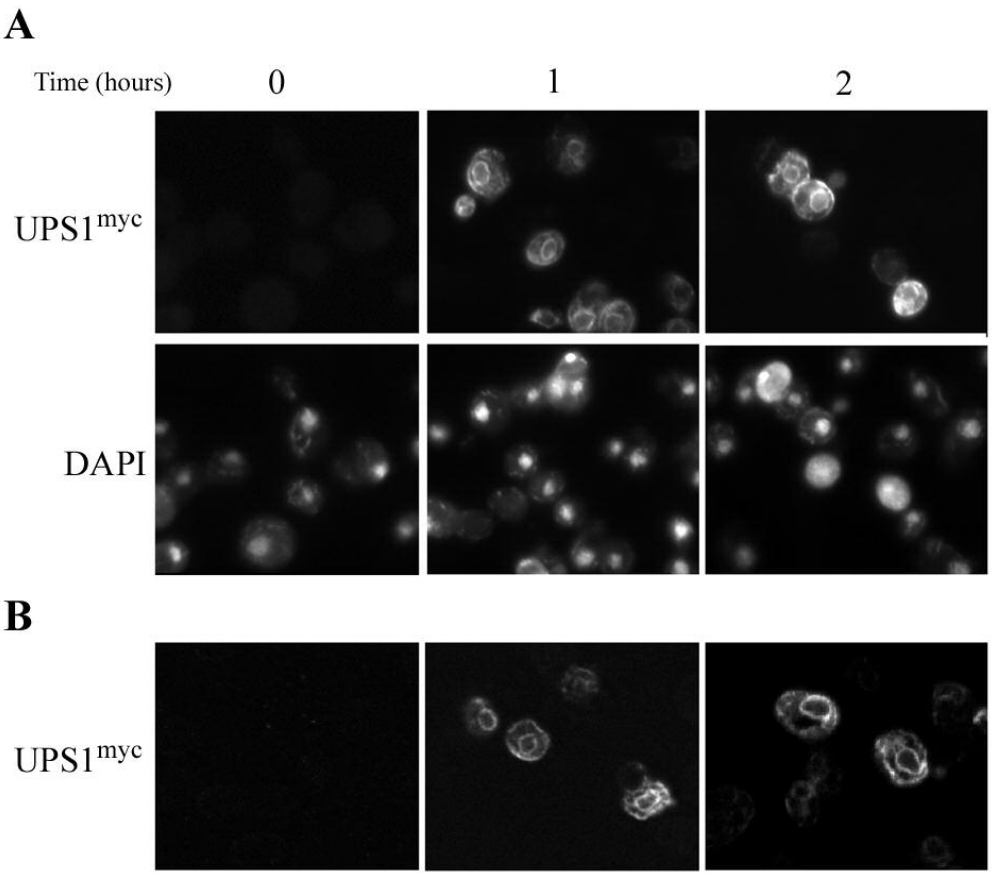
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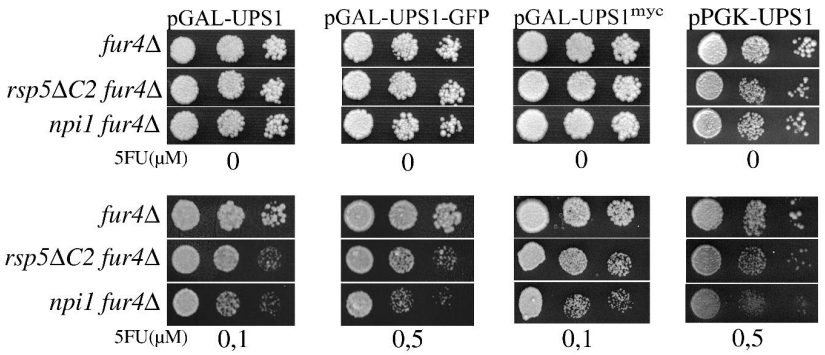
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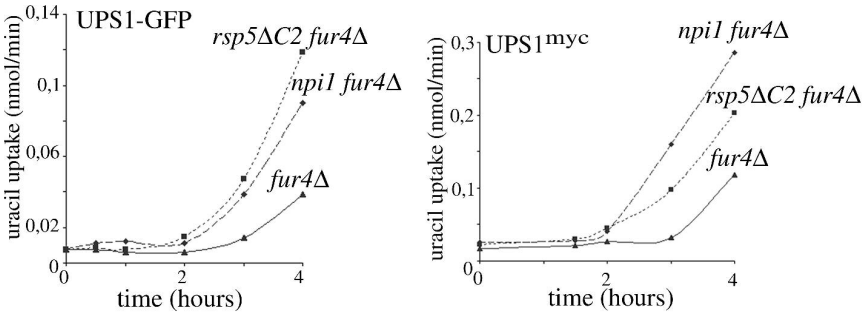
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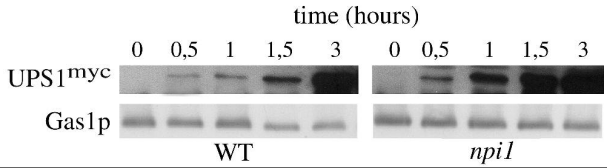
npi1 fur4Δ
fur4Δ
uracil uptake (nmol/min)
0,14
0,1
0,06
0,02
0
0
2
4
time (hours)
UPS1myc
npi1 fur4Δ
rsp5ΔC2 fur4Δ
fur4Δ
uracil uptake (nmol/min)
0,3
0,2
0,1
0
0
2
4
time (hours)

C


time (hours)
0
0,5
1
1,5
3
0
0,5
1
1,5
3
UPS1myc
Gas1p
WT
npi1